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Possible involvement of the inhibition of NF-kβ factor in anti-inflammatory actions that melatonin exerts on mast cells†

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ABSTRACT

Melatonin is a molecule endogenously produced in a wide variety of immune cells, including mast cells (RBL-2H3). It exhibits immunomodulatory, anti-inflammatory and anti-apoptotic properties. The physiologic mechanisms underlying these activities of melatonin have not been clarified in mast cells. This work is designed to determine the anti-inflammatory effect and mechanism of action of melatonin on activated mast cells. RBL-2H3 were pre-treated with exogenous melatonin (MELx) at physiological (100nM) and pharmacological (1mM) doses for 30 min, washed and activated with PMACI (phorbol 12-myristate 13-acetate plus calcium ionophore A23187) for 2h and 12h. The data shows that pre-treatment of MELx in stimulated mast cells, significantly reduced the levels of endogenous melatonin production (MELn), TNF-alpha and IL-6. These effects are directly related with the MELx concentration used. MELx also inhibited IKK/NF-kB signal transduction pathway in stimulated mast cells. These results indicate a molecular basis for the ability of melatonin to prevent inflammation and for the treatment of allergic inflammatory diseases through the down-regulation of mast cell activation. This article is protected by copyright. All rights reserved

KEY WORDS: Melatonin. RBL-2H3 mast cells. Cytokines. NF-kB. Inflammation.

Abbreviations used
MELx: Exogenous melatonin pre-treatment
MELn: Endogenous melatonin production
PMACI: Phorbol 12-myristate 13-acetate plus calcium ionophore A23187
NF-kB: Nuclear factor kappa B
MTT: 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
TNF-α: Tumor necrosis factor alpha
IL-6: Interleukin-six

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INTRODUCTION

Mast cells are cells of the immune system that are strategically located in places exposed to the environment, such as the skin, gut or airways, and hence these cells are ideal for the host defence [Abraham et al., 2010; Rich et al., 2013; Al-Azzam et al., 2015]. In order to perform these functions, mast cells express a variety of receptors that enable them to intervene in innate and adaptive immunity [Chen et al., 1998; Sandig et al., 2012]. These cells are dedicated to organising emergency inflammatory processes, in a few seconds, thanks to their proximity to the blood vessels [Rich et al., 2013]. These cells can be activated by both immunological and non-immunological means, eliciting the generation of a series of internal signalling pathways. These pathways include the activation of nuclear transcript factor NF-kB, which plays a key role in inflammation, defensive immune response, cell proliferation and apoptosis [Metz et al., 2007; Gilmore et al., 2011; Hosokawa et al., 2013; Sibilano et al., 2014]. During the inflammatory response, the nuclear transcript factor NF-kB induces the synthesis of MELn by immune competent cells via the inductions of enzyme arylalkylamine-N-acetyltransferase (AA-NAT) involved in the synthesis [Muxel et al., 2012; Villela et al., 2014]. This synthesized melatonin is crucial to modulate and regulate the functions of these immune cells, and this is performed with autocrine and paracrine effects [Muxel et al.,2012]. NF-kB is a protein complex composed of five members: RelA (p65), RelB, c-Rel, p50 and p52 and all these subunits may form dimers. RelA, RelB and cRel all have transactivating domains (TAD), while the other two (p50 and p52) have none. In the absence of stimulation, NF-kB is tethered in the cytoplasm by an inhibitor molecule, called IkB. When a mast cell receives extracellular stimuli, NF-kB is activated by means of the phosphorylation and degradation of IkB [Gosh et al., 1990]. The phosphorylation of IkB involves a specific IkB-kinase (IKK) complex, whose catalysis is generally carried out by three tightly associated IKK subunits (α, β and γ). IKK-α and IKK-β act as the catalytic subunits of the kinase. IKK-γ serves as the regulatory subunit [Zandi et al., 1997; Karin et al., 1999]. After its liberation, NF-kB inters the nucleus quickly and activates the expression of genes, which is involved in propagating the cellular response to inflammation. Therefore,

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the key steps for controlling NF-kB activity are: a) the regulation of the IkB-NF-kB interaction; and b) IkB-kinase activation [Sibilano et al., 2014; Gosh et al., 1990; Häcker et al., 2006]. Consequently, IkB degradation by activation of the IkB kinase could lead to the activation of the system. However, the exact mechanism by which melatonin modulates mast cell activation remains unclear; the objective of this study is therefore to investigate the effect of melatonin (MELx) on: the fitness cells, the production of TNF-α, IL-6 and MELn in activated mast cells, and finally to determine whether MELx could act by regulating NF-kB activity in mast cells.

MATERIALS AND METHODS

MATERIALS

Melatonin, luzindole, dimethyl sulfoxide (DMSO), Ca++ ionophore A23187 (CI) and phorbol 12-myristate 13-acetate (PMA), Tween 20, Deoxyribonuclease (DNase), ribonuclease (RNase), ammonium sulphate, EDTA/NA_2 , phenylmethylsufonyl fluoride and TritonX-100 were purchased from Sigma-Aldrich (St Louis, USA). DMEM Dulbecco´s modified Eagle´s medium and penicillin-streptomycin-glutamine were obtained from Gibco BRL, USA. Rabbit mAb to phospho-IKKalpha/beta (Ser176/180) (16A6) was obtained from Cell Signaling Technology.

PREPARATION OF MELATONIN AND LUZINDOLE

1M Melatonin and 10mM luzindole solutions were prepared in DMSO as the stock solutions and kept at –20ºC before use. Working solutions were prepared previously at room temperature by diluting the stock solutions with sterile DMEM to the desired concentrations. Those final concentrations of solvents were less than 0.5%.

MAST CELL CULTURE

The rat basophilic leukaemia mast cell line RBL-2H3 (ATCC 2256), is considered a good tool for the study of *in vitro* experimental models of mast cells [Passante et al., 2009; Funaba et al., 2003;

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Crivellato et al., 2015]. For this reason, they were chosen as the mast cells line for our study. The cells were maintained in DMEM supplemented with 15% heat-inactivated foetal bovine serum, 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin and amphotericin B solution at 37ºC under 5% CO2 in the air. Cells were used between passages 5 and 10.

MAST CELL AND PROTECTION BY MELATONIN

Mast cells were cultured (5 x 10^5cells/ml) in plates of 24 wells and were pre-treated with exogenous melatonin (MELx) at physiological (100nM) and pharmacological (1mM) doses for 30 min. Moreover, a non-specific melatonin receptor antagonist, luzindole 10µM, was co-incubated with 100nM and 1mM melatonin for the various experiments. The cells were then washed with PBS three times and a new medium with or without PMACI stimulus was administered.

MAST CELL ACTIVATION

Mast cells were subjected to PMACI stimulation, with and without pre-treatment of MELx. PMACI is formed by the union of PMA (phorbol-12-myristate 13-acetate) used at doses of 50 x 10^{-8}M and CI (calcium ionophore A23187) used at doses of 5 x 10^{-7}M, for 2 and 12 h. Previous pilot experiments of this study helped towards the selection of the times and concentrations of the combined PMA and CI for optimal cell stimulation in subsequent experiments [Maldonado et al; 2013]. Cell viability was determined by using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT assay; Sigma). After activation, cell free culture supernatants were collected, filtered, and stored at –20ºC for MELn and biological mediators, TNF-alpha and IL-6, determination. Likewise, the cells stimulated and unstimulated were collected from the wells, and the dry pellet was stored at -80ºC for subsequent studies.

MEASUREMENT OF CYTOKINES IN CULTURE SUPERNATANTS

TNF-alpha and IL-6 were chosen as cytokines representatives of the activation of mast cells,

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considering their documented role in the initiation of allergic inflammation and acute phase reactions [Maldonado et al., 2010; Lu et al., 2013]. TNF-alpha and IL-6 secretion was measured using a specific ELISA (BD OptEIATM). Capture antibody anti-rat TNF-alpha and IL-6 in 0.2M sodium phosphate, pH 9.0 were coated overnight to high binding microtiter plates. The plates were washed with phosphate buffered saline (PBS)/0.05% Tween 20 twice, incubated with 1% bovine serum albumin (BSA) in PBS/Tween 20 for 1h as a blocking step, and washed again. Samples and standards (recombinant TNF-alpha and IL-6) were diluted in 1% BSA PBS/Tween 20 and incubated overnight. After being washed five times, biotinylated anti-rat TNF-alpha or IL-6 mAb were added, and bound TNF-alpha or IL-6 detected using streptavidin-horseradish peroxidase conjugate, and 3,3´, 5,5´ tetramethylbenzidine and hydrogen peroxide as the substrate of the enzyme. The optical densities were determined at 450 nm in an automatic microplate reader. Both the intra- and inter-assay coefficients of variation were less than 10%.

MEASUREMENT OF MELn PRODUCTION IN CULTURE SUPERNATANTS

MELn content in the culture supernatants was assayed by ELISA kit (IBL- Hamburg Diagnostic, Germany) according to the manufacturers' instructions. This assay was based on the competition principle and microtiter plate separation. An unknown amount of antigen present in the sample and a fixed amount of enzyme labelled antigen competed for the binding sites of the antibodies coated onto the wells. After incubation, the wells were washed in order to halt the competition reaction. With the addition of p-nitro-phenyl phosphate (PNPP) substrate solution, the concentration of antigen was inversely proportional to the optical density measured. The measured ODs (450 nm) of the standards were used to construct a calibration curve (containing 0, 3, 10, 30, 100 or 300 pg/ml melatonin) against which the unknown samples were calculated. The lower limit of the assay was 3.0 pg/mL, and the intra-essay and inter-essay coefficients of variance were less than 10%.

WESTERN BLOT ANALYSIS

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In order to evaluate the level of protein in RBL-2H3 cells stimulated, unstimulated and pre-treated with MELx, the cells were lysed with extraction buffer (Hepes 50mM pH:7,5, EDTA 20mM, NaF 10mM, NaPPi 30mM, Benzamidina 2mM, Na3VO4 1mM, Tritón X-100 2%, protease inhibitor cocktail). Total lysates were subsequently incubated 20 min at 4º C. The soluble supernatants were denatured with loading buffer for 5 min at 95º C, resolved by SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked for 1h at room temperature in PBS-T (PBS with 0.2% of Tween 20) containing 3% of BSA. The blots were then incubated for 3h at room temperature with rabbit mAb to phospho-IKKalpha/beta (Ser176/180) (16A6) (Cell Signaling Technology). Blots were washed in PBS-T 3 times, exposed to horseradish peroxidise-coupled antirabbit immunoglobulin, and detected by hyperfilm ECL by using a western blotting luminol reagent from Santa Cruz Biotechnology.

STATISTICAL DATA ANALYSIS

The results were expressed as arithmetic means ± standard error of the mean (S.E.M.) in the numbers of determinations and were analysed statistically by means of one-way ANOVA followed by Tukey-Kramer multiple comparison test and Student's paired t-test, with the level of significance set at $p<0.05$.

RESULTS

RELEASE OF PRO-INFLAMMATORY CYTOKINES BY RBL-2H3 CELLS IN RESPONSE TO PMACI

As shown in Figure 1, RBL-2H3 cells produced substantial amounts of TNF-alpha and IL-6 after stimulation with PMACI for 12h. The data indicated that PMACI activated RBL-2H3 cells in a concentration-dependent manner. Thus, the levels of TNF-alpha were 82% higher in stimulated than in unstimulated cells (Figure 1). Similar results were obtained for IL-6; the only difference being that the rate of IL-6 was about 68% higher with stimulation than without it (Figure 1). We also

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observed an increase in the synthesis and release of melatonin (MELn), by the own mast cells, as another cytokine more. The MELn released in the total medium, after 12h of incubation with PMACI, can be examined in Figure 3. It can be observed, how unstimulated cells release MELn and how the PMACI stimulation increased this secretion by 63%. Insignificant amounts of melatonin were detected in the medium alone and in the medium plus DMSO (control vehicle).

EFFECTS OF MELx ON THE RELEASE OF PRO-INFLAMMATORY CYTOKINES BY RBL-2H3 CELLS IN RESPONSE TO PMACI

In order to determine whether exogenous melatonin pre-treatment (MELx) can modulate PMACI-induced TNF-alpha and IL-6, cells were pre-incubated with various concentrations of MELx (data not shown); the final selection was the physiological and pharmacological doses of 100nM and 1mM, for 30 min. Then, washed and activated with PMACI. Culture supernatants were assayed, after 12h, for TNF-alpha and IL-6 by the ELISA method. MELx 100nM reduced the secretion of TNF-alpha and IL-6 in stimulated mast cells with inhibitory effects of 30%, and 40%, respectively. This inhibition was more marked at higher MELx concentrations. Thereby, with MELx 1mM the inhibitory effects were of 60% and 55% respectively (Table 1 and Figure 1).

EFFECTS OF MELx ON THE RELEASE OF MELn BY RBL-2H3 CELLS IN RESPONSE TO PMACI

In order to determine whether exogenous melatonin pre-treatment (MELx) can modulate PMACI-induced endogenous melatonin production (MELn), the cells were pre-treated with MELx 100nM, 1mM and luzindole, a non-selective competitive melatonin antagonist receptor [Rivara et al., 2005], for 30 min. Then, washed and activated with PMACI. Culture supernatants were assayed, after 12h, for MELn by ELISA method. As can be observed in Table 1 and Figure 3, the pre-treatment with MELx, 100nM and 1mM, in stimulated mast cells, reduced the synthesis and the release of MELn by 24% and 33%, with statistically significant values *$p<0.05$ and ***$p<0.001$ respectively. When

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the antagonist luzindole was used concomitantly with the MELx pre-treatment, the effects of MELx 100nM and 1mM on the synthesis and release of MELn by mast cells were blocked, although not entirely for MELx 1mM (*$p<0.05$).

EFFECTS OF MELx ON THE RELEASE OF CYTOKINES AND MELn BY RBL-2H3 CELLS, WITHOUT STIMULATION

When the RBL-2H3 cells were pre-treated with MELx for 30 minutes, washed and maintained in culture for 12 h without stimulation, they showed no statistically significant differences with regard to the pro-inflammatory cytokines (TNF-α and IL-6) and MELn from the RBL-2H3 control (non-pre-treated and non-stimulated), (Figure 2).

EFFECTS OF MELx ON THE CYTOTOXICITY GENERATED BY THE CHEMICAL STIMULATION ON RBL-2H3 CELLS

PMACI chemical stimulation triggered a state of hyper-stimulation in the mast cells that led to cellular apoptosis [Maldonado et al., 2013]. Cell viability was determined by using the MTT assay. Figure 4 show that the exposure to PMACI for 12h reduced the cell viability in a dose-dependent manner. This was reflected by a decline in the statistically significant fitness cells (***$p<0.001$) between stimulated and unstimulated cells.

Mast cells pre-treated with MELx to physiological and pharmacological doses, (100nM and 1mM) respectively, elevated the rates of cell viability thereby improving the physiological, original normality of the cells. The treatment with the antagonist luzindole reversed the cytoprotection by MELx. Specifically, Figure 4 shows how the effect of MELx 1mM on cellular cytoprotection, subject to 12h of chemical stimulus, is partially blocked by the melatonin receptor antagonist luzindole, with statistically significant values for MELx 1mM (*$p<0.05$).

EFFECTS OF MELx ON NF-KB SIGNALLING IN MAST CELLS

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In order to investigate the intracellular mechanism responsible for the effects of MELx on pro-inflammatory cytokine production and cytoprotection in stimulated mast cells, we examined the effect of MELx on the activation of nuclear factor-kB (NF-kB) by using the Western blot analysis. Stimulation of RBL-2H3 cells, with PMACI for 2h, induced the phosphorylation of IkB. The pre-treatment with MELx, at physiological 100nM (10^{-7}M) and pharmacological 1mM (10^{-3}M) doses, interfered, at least partly, with the phosphorylation induced by the activation of IKKα/β (Figures 5 and 6).

DISCUSSION

Several agents may activate the nuclear factor-kappa B (NF-kB) by different cellular mechanisms including oxidant stress, mitogens, viral infections, cytokines and PAMPs [Mohan et al., 1995; Baeuerle et al., 1997]. The PMACI complex formed by the union of PMA (phorbol 12-myristate 13-acetate) and Calcium ionophore A23187 can induce NF-kB activation by the action of both compounds in mast cells [Mohan et al., 1995; Manna et al., 1998; Cikler et al., 2005]. For this reason, PMACI has been used as a direct and convenient reagent to examine the mechanism underlying inflammatory and allergic reactions in mast cells [Kim et al., 2006]. The activation of mast cells by PMACI leads to the dissociation of NF-kB / IkB complex and migration of NF-kB to the nucleus, promoting the cytokines synthesis [Gosh et al., 1990; Mohan et al., 1995; Kim et al., 2014]. An increase in NF-kB activity associated with the secretion of high levels of TNF-alpha and IL-6 has also been noticed in the context of allergic inflammatory responses [Trefzer et al., 2003; Kim et al., 2013]. TNF-alpha and IL-6 are pro-inflammatory cytokines that mediate multifunctional responses including cell survival, growth, differentiation, and the defensive immune response in mast cells [Lippert et al., 2000]. Therefore, the focus of the production of TNF- alpha and IL-6 by mast cells can aid in the development of a useful therapeutic strategy for allergic inflammatory diseases [Lippert et al., 2000; Kim et al., 2013]. In this end, we investigate the effects of MELx on PMACI-induced cytotoxicity and TNF-alpha, IL-6 and MELn production in RBL-2H3. In order to

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address how MELx improved cellular fitness and inhibited TNF-alpha, IL-6 and MELn production, we focus on the signalling pathway NF-kB in mast cells. As a result, we find that PMACI-stimulated mast cells enter into a state of hyperactivity, which is revealed by a decrease in cell viability and by the release of cytokines, such as TNF-alpha, IL-6 and MELn, into the medium (Figures 1, 3 and 4). However, the mast cells pre-treated with MELx (100nM and 1mM), 30 min. before PMACI stimulus, improve cell viability and significantly reduce the level of production of TNF-alpha, IL-6 and MELn. The effect is dose-dependent, with optimal results at physiological (100nM) and pharmacological (1mM) doses (Figures 1, 3 and 4). Thus, the inhibitory effect of MELx on TNF-alpha and IL-6 production, by activated mast cells, is of 30% and 40% for the physiological dose, and of 60% and 55%, respectively, for the pharmacological dose (Table 1). Amazingly, our results show (Figure 3) how unstimulated mast cells, also secreted MELn and how stimulation with PMACI raised the production by 63%. It has been proposed that mast cells constitutively synthesize and release MELn as an immunoregulatory molecule involved in the maintenance of cellular oxidant–antioxidant homeostasis or timing of pro- and anti-inflammatory effects, in forced and normal physiology of the cells [Galli et al., 1996, 2005; Maldonado et al., 2010]. These results suggest that exogenously added melatonin may interfere with endogenously generated melatonin. However, the effects of MELx on the production MELn and proinflammatory cytokines in unstimulated cells remained insignificant, compared to those of control cells (untreated and unstimulated) (Figure 2). It seems that the effect of MELx on mast cells is only relevant in situations of damage or danger cellular. Mast cells could act as injury sensors, detecting cellular risk or insecurity, and exacerbate inflammation through releasing additional of MELn (Enoksson et al., 2011).

As in other cells, mast cells stimulation activates the NF-kB signalling pathway, which plays an important role in the MELn synthesis by inducing the activation of the enzyme Arylalkylamine-N-Acetyltransferase (AA-NAT) [Pontes et al., 2006; Muxel et al.,2012; Hu et al., 2013; Markus et al.,

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2013; Vilella et al., 2014]. The same group [Pontes et al., 2006; Markus et al., 2013] raised the hypothesis that the transcription of the gene that codifies AA-NAT could be under NF-kB control, since kB elements are present in the promoter and in the first intron [Markus et al., 2007]. This was more recently confirmed by controlling the expression of a red protein with part of the promoter and the first intron of this gene in monocyte cells [Markus et al., 2013; Pires-Lapa et al., 2013]. The authors clearly show that when the nuclear translocation of NF-kB is induced by different stimuli, both the red reporter and AA-NAT gene are transcribed and melatonin is detected in the culture medium. The same could be happening with RBL-2H3 cells. PMACI activates the nuclear translocation of NF-KB, which increases the transcription of MELn. This would explain the levels of MELn found in the supernatant of mast cells under the influence of the PMACI (Figure 3). The ability of mast cells to synthesize/release melatonin and express MT1 and MT2, two membrane receptors of melatonin, has been well documented in our previous work; this ability is, especially true after activation [Maldonado et al., 2010]. The presence of MT1 and MT2 in the membrane of unstimulated mast cells and their raised expression after the stimulus, confirms our hypothesis concerning the role of MELn as a restorer of the physiological balance inside and outside the mast cell.

In order to evaluate whether the state of hyperactivity/apoptosis generated by PMACI on mast cells is improved with the pre-treatment of MELx, we measure cell viability using MTT assay. The results in Figure 4 show that pre-treatment with MELx exercised cytoprotection on mast cells and this was better in pharmacological doses (1mM) than in physiological doses (100 nM). Supporting this idea, the luzindole, a non-selective melatonin receptor antagonist, blocked their protection by 1mM melatonin, but not completely. This may well be due to the lingering presence of MELx in mast cells, which renders any cytoprotection, via melatonin receptor independent [Reiter et al., 2007; Maldonado et al., 2013].

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Since the NF-kB pathway is a multi-component pathway, there are several methods and steps to inhibit NF-kB activation [Zhang et al., 2015] (Figure 6). Activation of NF-kB by pro-inflammatory stimuli involves phosphorylation on Ser 177 and 181 for IKKβ, and Ser 176 and 180 for IKKα [Mercurio et al., 1997; Ling et al., 1998]. According to our results, RBL-2H3 cells stimulated with PMACI, presented a deep induction of IKKα phosphorylation compared to the unstimulated cells (Figure 5). Here we show that pre-treatment with MELx before the PMACI stimulation, at concentrations of 100nM and 1mM, prevents the up-induction of IKKα phosphorylation and effects on the IKK/IkB cascade would, in turn, contribute to inhibition of NF-kB activation. Inhibitory effects of melatonin on NF-kB-binding activity have been previously found in many cells and tissues, such as macrophages [Gilad et al., 1998], muscle tissue [Alonso et al., 2006], hepatocytes [Liang et al., 2009], or neurons [Ali et al., 2015], this inhibitory effect may play a role in the amelioration of the inflammatory damage [Li et al., 2005]. However, we only documented that melatonin can reduce the phosphorylation of IKKα. While an extensive structural homology between catalytic subunits of IKKα and IKKβ, and their similar abilities to phosphorylate IkB proteins suggest that their functions are likely to be redundant and overlapping; some site-directed mutagenesis experiments propose that IKKα and IKKβ may possess distinct regulatory functions [Gosh et al., 2002; Olivotto et al., 2014]. The two pivotal kinases IKKα and IKKβ activate NF-KB dimers that might translocate to the nucleus and regulate the expression of specific target genes. The IKKβ-driven canonical NF-KB signalling pathway orchestrates most stress/inflammatory response and modulates, among others, inflammation, apoptosis and proliferation. The non-canonical NF-KB signalling pathway, controlled by IKKα, mediates adaptative immunity and participates in cell survival and differentiation processes in different cell types. Hence, the two IKK catalytic subunits are involved in complimentary roles needed for the control of inflammation [Olivotto et al., 2014].

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In conclusion, this study reveals that PMACI induces the activation of NF-kB and causes an inflammatory toxicity on mast cells, which could be mitigated by MELx. This mitigation is shown by the improvement of cell viability and the decrease of TNF-alpha, IL-6 and MELn levels.

CLINICAL IMPLICATIONS

Melatonin could be used as a therapeutic agent in inflammatory processes and allergic diseases due to two of its actions: a) it attenuates the activation of NF-kB, thereby reducing the production of TNF-alpha and IL-6; and b) it generates cytoprotection, thereby promoting the survival of mast cells.

Conflict of interest

The authors declare this research has been conducted in the absence of any commercial or financial relationships that could be considered as a potential conflict of interest.

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Table 1: Inhibition (%) of TNF-alpha, IL-6 and MELn from RBL-2H3 mast cells subjected to 30 min. of pre-incubation with MELx (100nM and 1mM) before chemical stimulation with PMACI for 12h. Dates are expressed as mean percentage ± SEM of 6 experiments.

Citokines secretion	Therapy Melatonin	% Inhibition 30 min. Pre-incubation
TNF- α	100nM	30% ± 2.8%
	1mM	60% ± 1.7%
IL-6	100nM	40% ± 3.3%
	1mM	55% ± 2%
MELn	100nM	24% ± 3.9%
	1mM	33% ± 2.5%

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Figures:

Fig. 1. Inhibitory effect of MELx on the production of pro-inflammatory cytokines, TNF-alpha and IL-6, induced by PMACI. Each bar represents the means ± SEM for six separate experiments and each sample was analysed in triplicate. C*: stimulated cells; MELx: exogenous melatonin treatment.

Fig. 2. There was no significant difference (NS) in pro-inflammatory cytokines (TNF-alpha, IL-6 and MELn) between control cells (RBL-2H3 without treatments and without stimulation) and cells pre-treated and unstimulated. Each bar represents the percentage of change in TNF-alpha, IL-6 and MELn release relative to control cells.

Fig. 3. Inhibitory effect of MELx on synthesis and release of MELn induced by PMACI. Each bar represents the means ± SEM for six separate experiments and each sample was analysed in triplicate. Control: medium alone; Control vehicle: medium alone plus DMSO; C: unstimulated cells; C*: stimulated cells; MELx: exogenous melatonin treatment; LZD: luzindol.

Fig. 4. Cytoprotective effects of MELx, 100nM and 1mM doses, on RBL-2H3 cells activated by PMACI for 12h. Each bar represents the means ± SEM for four separate experiments and each sample was analysed in triplicate. C: unstimulated cells; C*: stimulated cells. MELx: exogenous melatonin treatment; LZD: luzindole.
* $p<0.05$; *** $p<0.001$.

Fig. 5. Inhibitory effect of MELx on IKKα/β phosphorylation induced by PMACI in RBL-2H3 cells. Mast cells were pre-incubated with 100nM and 1mM of MELx for 30 min., washed and prepared for stimulation with PMA 5. 10-7 M + CI 5. 10-7 M, for 2h. The effect was studied using Western blot analysis.

Figure 6. Schematic proposal diagram showing the interaction between acute inflammation and the anti-inflammatory action that melatonin would exert on mast cells.

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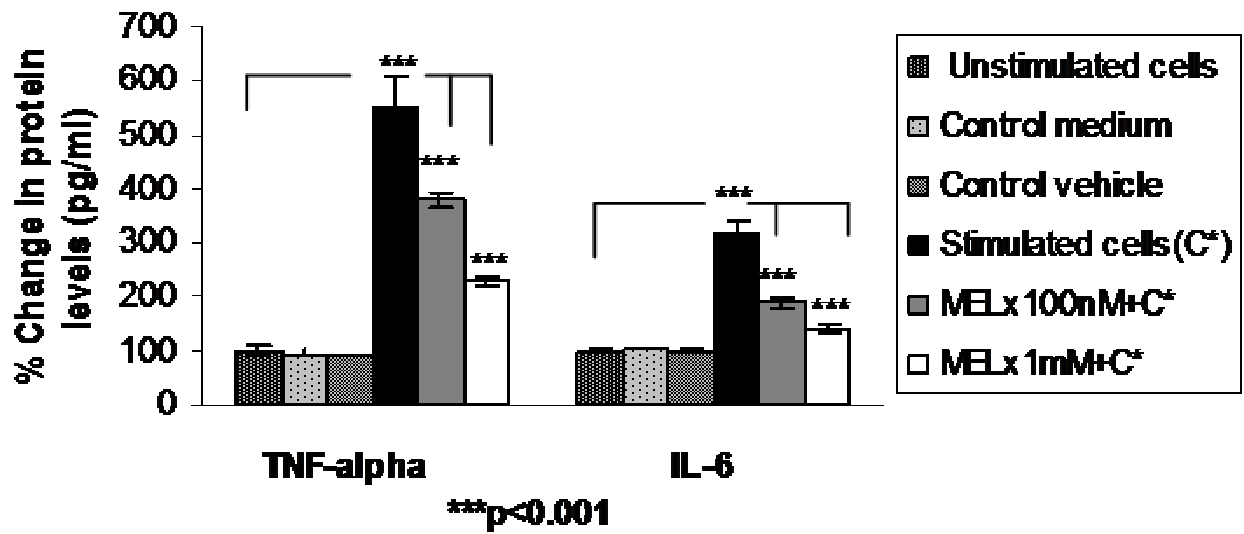

Figure 1

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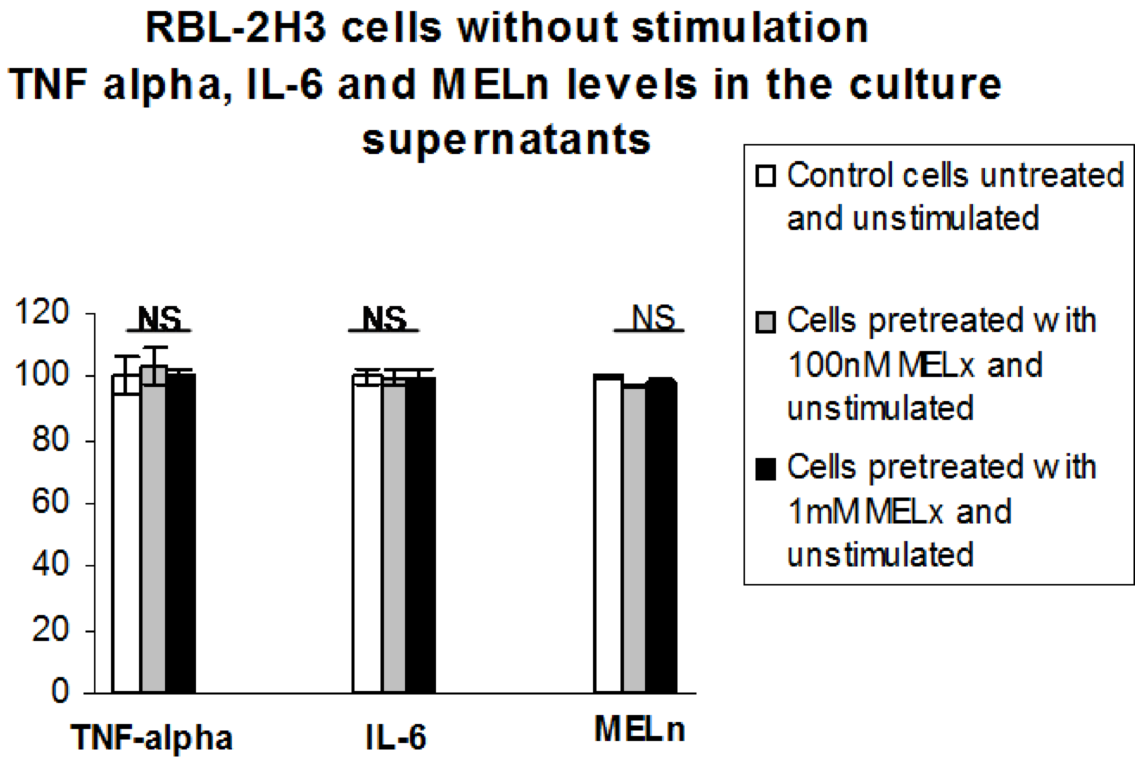

Figure 2

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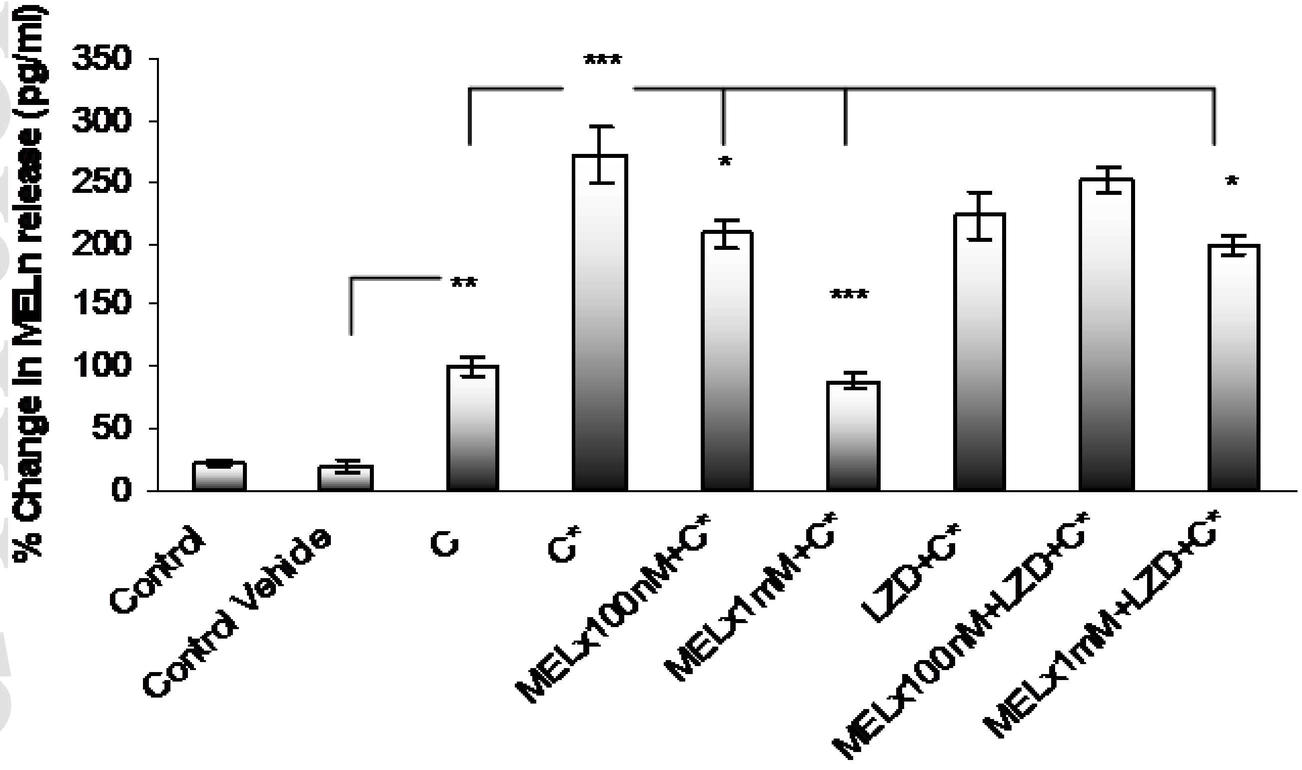

Figure 3

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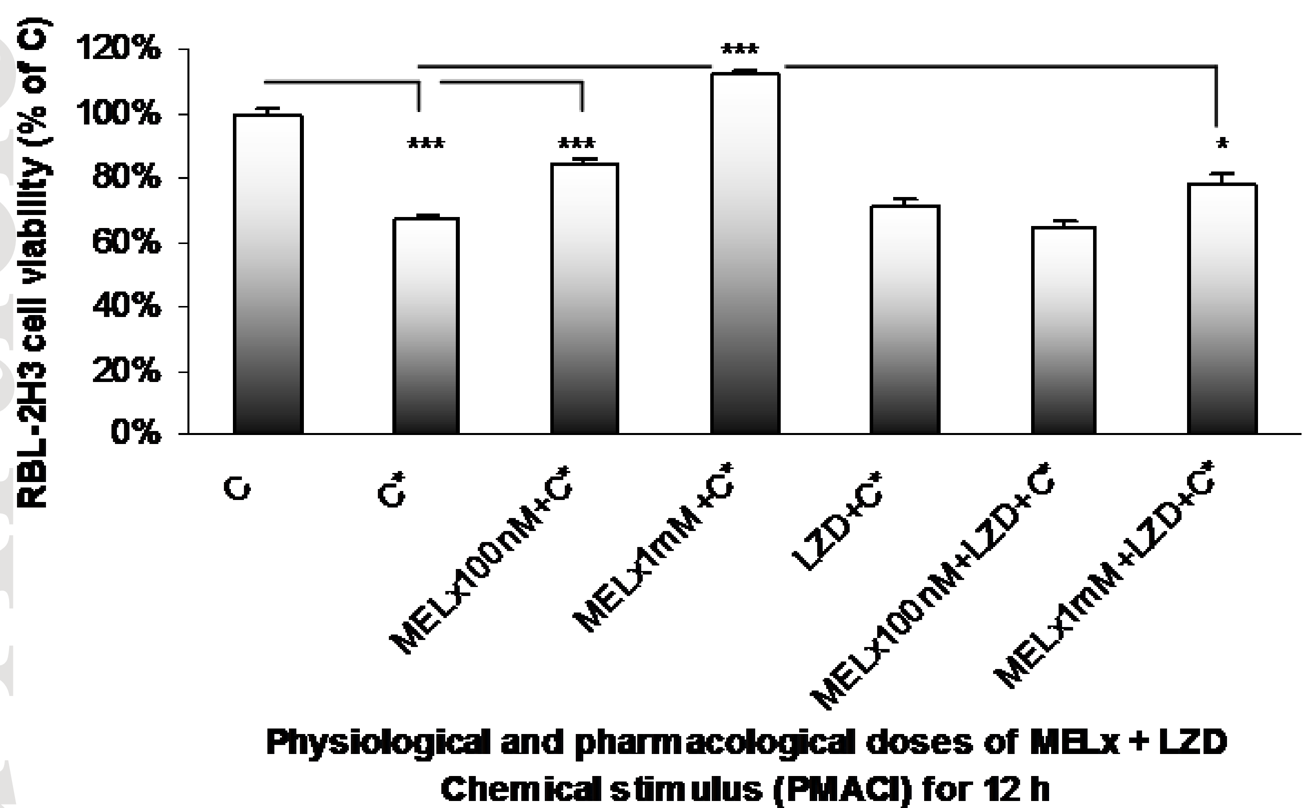

Figure 4

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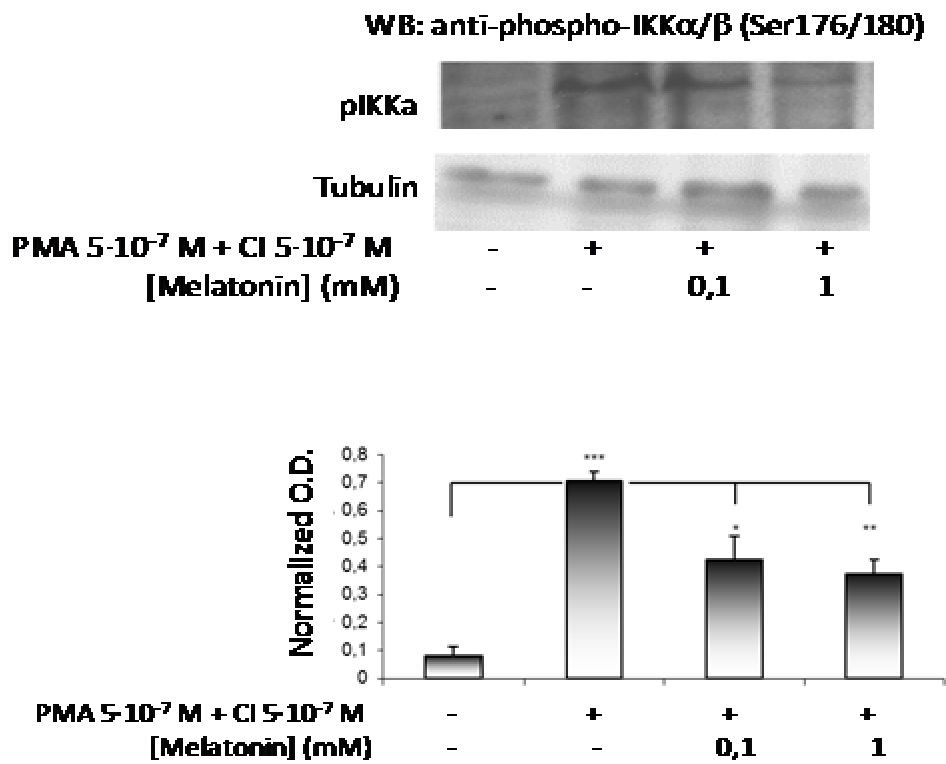

Figure 5

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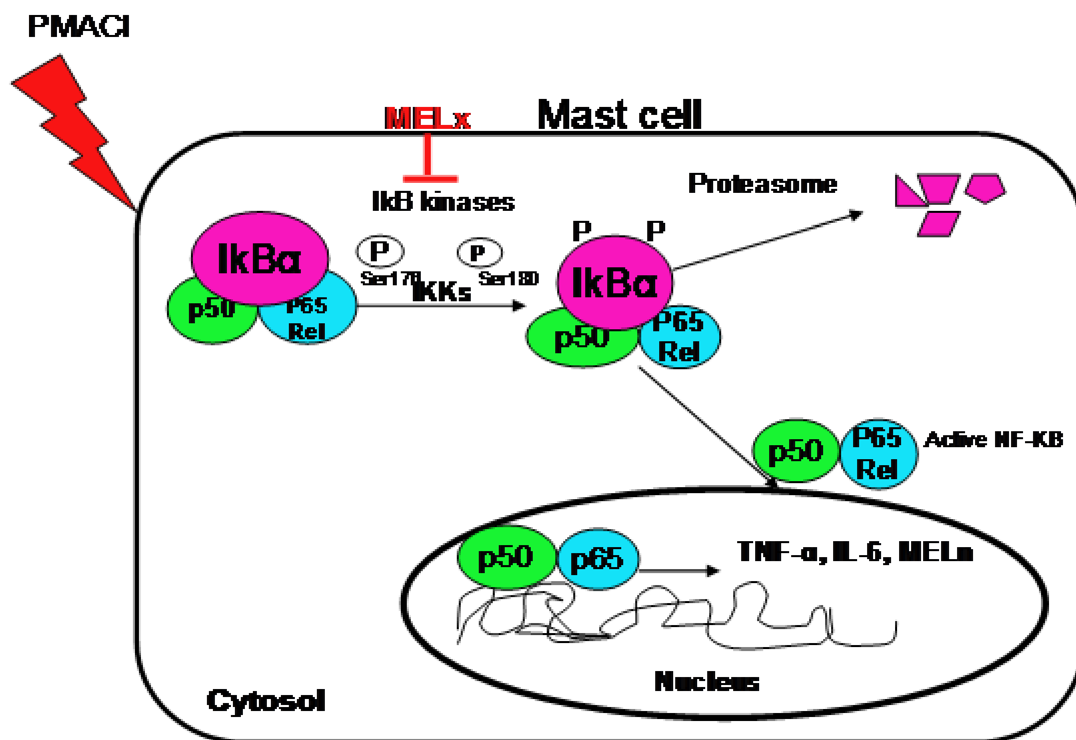

Figure 6

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